JOURNAL OF MEDICAL VIROLOGY

VOLUME 59
NUMBER 1
SEPTEMBER 1999

WILEY-LISS
ISSN 0146-6615

Journal of Medical Virology 59:60–65 (1999)

Adeno-Associated Virus and Development of Cervical Neoplasia

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Evidence from several sources has suggested that adeno-associated virus (AAV) infection might protect against cervical cancer, in part, by interfering with human papillomavirus (HPV)-induced tumorigenesis. Detection of AAV type 2 (AAV-2) DNA in cervical tissues has been reported. However, there have been few in vivo studies of women with cervical HPV infection or neoplasia, and these have reported inconsistent results. Therefore, we used polymerase chain reaction (PCR) assays targeted to the AAV-2 *rep* and *cap* genes to test tissue specimens from women in an epidemiological study of cervical neoplasia in Jamaica. We tested 105 women with low-grade cervical intraepithelial neoplasia (CIN-1), 92 women with CIN-3/carcinoma in situ or invasive cancer (CIN-3/CA), and 94 normal subjects. PCR amplification of human β-globin DNA was found in almost all cervical specimens, indicating that these materials were adequate for PCR testing. The prevalence of HPV DNA, determined by HPV L1 consensus primer PCR was, as expected, strongly associated with presence and grade of neoplasia. Each of the AAV PCR assays detected as few as 10 copies of the virus genome. However, none of the 291 cervical specimens from Jamaican subjects tested positive for AAV DNA. Negative AAV PCR results were also obtained in tests of cervical samples from 79 university students in the United States. Exposure to AAV was assessed further by serology. Using a whole virus AAV-2 sandwich enzyme-linked immunosorbent assay, we found no relationship between AAV antibodies and presence or grade of neoplasia in either the Jamaican study subjects or women enrolled in a U.S. study. Overall, the data provide no evidence that AAV infection plays a role in cervical tumorigenesis or that AAV commonly infects cervical epithelial cells. ***J. Med. Virol. 59:60–65, 1999.***

KEY WORDS: adeno-associated virus; AAV; cervical cancer; CIN; human papillomavirus; HPV

INTRODUCTION

Increasing evidence has suggested that infection with adeno-associated virus (AAV) might protect against development of cervical neoplasia. AAV is a helper-dependent parvovirus, requiring coinfection with other DNA viruses, such as adenovirus, for its replication [Leonard and Berns, 1994]. In tissue culture, AAV-2 inhibits the transformation of cells by human papillomavirus (HPV) [Hermonat, 1994b], the sexually transmitted DNA virus linked etiologically to cervical tumorigenesis [Levine et al., 1998]. AAV-2 similarly interferes with cell immortalization by bovine papillomaviruses (BPV) [Hermonat, 1989] and suppresses papillomavirus replication [Hermonat, 1992]. HPV was shown recently to support replication of AAV-2 [Walz et al., 1997], making it plausible that the two viruses could be associated in nature. In addition, AAV may have antineoplastic effects through the direct

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interaction of AAV proteins with cellular genes. In tissue culture, AAV induces differentiation of tumor cell lines [Bantel-Schaal, 1995], down-regulates *c-fos* and *c-myc* [Hermonat, 1994a], inhibits cell proliferation [Walz and Schlehofer, 1992], and reduces carcinogen-induced mutagenicity [Schlehofer and Heilbronn, 1990].

These laboratory findings have added credence to observational, seroepidemiologic studies conducted years earlier, suggesting an inverse association between AAV infection and development of cervical neoplasia, as well as other neoplasms. In the initial case-control studies by Sprecher-Goldberger et al. [1971], AAV-3 seroprevalence, as determined by complement fixation assays, was lower in cancer cases relative to matched controls: 18% vs. 32% for cervical cancer; 14% vs. 22% in breast cancer; 22% vs. 43% in lung cancer; and 17% vs. 44% in leukemia [Sprecher-Goldberger et al., 1971]. Mayor et al. [1976], using an immunofluorescence assay, also found low prevalence of AAV-2/3 antibodies among cervical cancer patients (30% vs. 70% in controls). Low titers of anti-AAV-5 immunoglobulin G (IgG) but not low seroprevalence in cervical cancer patients (60% vs. 68% in controls) were observed by Georg-Fries et al. [1984], using an enzyme linked immunosorbent assay (ELISA). Recently, Tobiasch et al. [1994] found decreased prevalence of AAV-2 IgM, but not IgG, in patients with precancerous lesions of the cervix, called cervical intraepithelial neoplasia (CIN).

If correct, the putative antineoplastic effects of AAV in the cervix must involve direct infection of cervical epithelial cells with AAV and less frequent infections among women with severe cervical neoplasia. Consistent with this assumption, Walz et al. [1997] detected DNA from AAV-2, the most common AAV type, by polymerase chain reaction (PCR) in 21 (78%) of 27 normal cervical specimens, 8 (47%) of 17 CIN-II/III, and 4 (44%) of 9 invasive cervical cancers (a statistically significant trend, $P = .03$, by our calculations). In an earlier study, the same laboratory detected AAV-2 in 8 (80%) of 10 normal cervical specimens, 6 (60%) of 10 CIN lesions, and 3 (60%) of 5 invasive cervical cancers [Tobiasch et al., 1994]. Most recently, this laboratory reported isolation of infectious AAV from cervical specimens [Walz et al., 1998]. However, these observations were not confirmed in a recent investigation. Friedman-Einat et al. [1997] detected AAV-2 DNA in none of 21 HPV-containing cervical biopsy specimens, and in only 1 (2%) of 61 genital swabs from women with suspected herpes simplex virus following 35 cycles of AAV PCR amplification (18% were positive after 70 PCR amplification cycles).

We examined this issue by testing specimens obtained during an epidemiological study of cervical neoplasia in Jamaica. No AAV DNA was detected in any cervical specimens, despite using sensitive PCR assays targeted to two separate regions of the AAV-2 genome. Serology also failed to suggest a relation of AAV-2 exposure with presence or grade of neoplasia. The negative DNA hybridization results were confirmed in cervical specimens collected from university students in the U.S., and the null serologic results were confirmed in specimens from a U.S.-based case-control study of invasive cervical cancer.

METHODS

Subject Enrollment and Specimen Collection

Jamaican subjects. We studied 105 women with CIN-1, 92 women with either CIN-3/carcinoma in situ ($n = 81$) or invasive ($n = 11$) cervical cancer (CIN-3/CA), and 94 normal controls. All subjects were selected from participants in an epidemiological study of cervical neoplasia in Jamaica. Selection of women with cervical neoplasia was conducted using stratified random sampling to choose among sequential colposcopy patients ($n = 447$), 20–49 years of age, enrolled at their presentation to the University of the West Indies colposcopy clinic, Kingston, Jamaica (November 1994 through April 1998). Women with normal cytology were selected from among sequential patients ($n = 500$) who attended the affiliated cervical cancer screening clinic (Spring 1996 and Spring 1997), by frequency age-matching these controls to CIN-3/CA cases. During enrollment, a cervical Pap smear was prepared for each patient, using an Ayre spatula and endocervical cytobrush. The Ayre spatula and cytobrush were then each placed into PreservCyt (Cytyc Corp., Marlborough, MA) media to collect remaining cervical cells for later production of monolayer ThinPrep cytological specimens (Cytyc). To obtain cervical cells for virologic testing, after the Pap smear a dacron swab was used to collect samples from the ecto- and endocervix, which were placed immediately in Virapap transport medium (Digene Diagnostics, Silver Spring, MD). Peripheral blood samples were obtained and separated into serum and cellular material. All serum and Virapap specimens were stored at −70°C until tested. Among colposcopy patients, all acetowhite lesions observed under colposcopic magnification were biopsied and fixed in formaldehyde for histopathologic evaluation. Conventional cyto/histopathology was performed by a single research pathologist (CE) and ThinPreps were assessed independently by a single cytologist (MH) expert in their evaluation. Patient diagnoses were assigned according to the highest grade of neoplasia. Only subjects whose several pathology findings were all consistent within one grade of neoplasia were selected for the current investigation.

University of Maryland students. Undergraduate and graduate women ($n = 79$) aged 18–40 years who presented to the University Health Center and received routine annual pelvic examinations were enrolled sequentially during 1996. The specimen collection procedures have been reported previously and were similar to above [Kotloff et al., 1998], except that no PreservCyt specimens were obtained. All patients except for one patient with CIN-1 had normal cytological findings.

U.S. cervical cancer patients and controls. The National Cancer Institute (NCI) Immunodiagnosis Serum Bank, established by NCI through contract with the Mayo Clinic, provided sera from hospitalized patients with cervical cancer ($n = 74$) and hospitalized noncancer controls ($n = 77$), frequency age-matched to the cases. The specimens tested were collected by the serum bank between 1975 and 1991, as part of a broader project to obtain blood specimens from inpatients diagnosed with 85 different types of cancers, benign tumors, and nontumor-related conditions. All sera were stored at −70°C until tested [DiMagno et al., 1989].

Laboratory Procedures

DNA hybridization methods.

DNA extraction for AAV PCR. After defrosting, 500 μl of each Virapap specimen was transferred to a 1.5-ml Eppendorf tube. To each sample, one drop of digestion buffer (Digene Diagnostics) was added, vigorously mixed, and allowed to react for 2 hr at 37°C. One hundred microliters of each digest were then ethanol precipitated, dried, and resuspended in 100 μl of 10 mM Tris pH 7.5 with 1 mM ethylenediamine tetraacetic acid (EDTA), before amplification or refreezing at −70°C.

AAV PCR. We selected two sets of primers, reported previously to detect AAV in cervical specimens, and targeted to separate regions of the AAV genome. The first primer set, described by Han et al. [1996], 5′-CATCGCGGAGGCCATAGCCC/5′-ACGGGAGTC-GGGTCTATCTG (HAN-1/HAN-2), amplifies a 221-bp region of the AAV *rep* gene. Primers A7/A8 amplify a 369-bp region from the AAV-2 *cap* gene [Grossman et al., 1992; Friedman-Einat et al., 1997]. To 10 μl of each digested specimen, 40 μl of PCR buffer were added, yielding a final concentration of 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTP, 0.5 μM of each primer, and 2.5 units of *Taq* polymerase. Forty amplification cycles were performed using 95°C for 20 sec, 55°C for 30 sec, 72°C for 30 sec, and on the last cycle, 5 min for extension at 72°C. The products of these amplifications were denatured, drawn onto a nylon membrane (Biotrans ICN, Inc., Cosa Mesa, CA), and immobilized by ultraviolet light on a transilluminator.

Filters were hybridized at 55°C in 5× saline-sodium phosphate-EDTA (SSPE) with 0.1% sodium dodecyl sulfate (SDS) and then washed in 2× SSPE with 1% SDS, also at 55°C. Hybridization was performed with biotinylated probes using the Amersham enhanced chemiluminescence system according to the manufacturer's instructions (Amersham Corp., Arlington Heights, IL). Oligonucleotide probes were selected from sequences located between the two PCR primers in each assay. Probe for HAN-1/HAN-2 PCR was TG-GTGGGAGGAGGGGAAGATGACC, and for A7/A8 PCR it was AGGAAAACAGCAAACGCTGG. To assess the analytic sensitivity of the PCR assays, and to act as positive controls, on every plate we included 10-fold titrations of an AAV-2 plasmid (ATCC #37215) in [illegible]

Negative controls on every plate were DNA specimens extracted from suspensions of K562 cells.

HPV PCR. Testing for HPV DNA was conducted under conditions described previously [Rattray et al., 1996; Kotloff et al., 1998; Strickler et al., 1998], using MY09/MY11 consensus primers that amplify a conserved 450-bp region from the L1 open reading frame, with consensus probes (GP1 and GP2), as well as type-specific probes for more than 15 of the most common anogenital HPV types.

β-globin PCR. To assess the adequacy of tissue extracts for PCR, each specimen was tested for amplification of a 268-bp region of the human β-globin gene, using primers GH20/PC04 and probe PC03, under the same assay conditions used during AAV and HPV testing.

ELISA.

AAV-2 particle preparation. In replicate flasks, 5 × 10^7 HeLa S3 cells were grown in 50 ml culture medium (Dulbecco's Modified Eagle Medium, Gibco, Grand Island, NY with 10% fetal calf serum). When cells reached 50–60% confluency, the cultures were seded with Adenovirus type 2 (ATCC #VR-846) at 5–10 MOI and with AAV-2H (ATCC #VR-680) at 10–20 MOI for 1 hr at 37°C. The infected culture supernatants were then replaced with fresh medium. Production of AAV was confirmed on days 2–3 following inoculation by immunofluorescence with anti-AAV guinea pig antiserum (Braton Biotech, Rockville, MD #V-267-502-558), and the cells were harvested. Virus was released from cells using repeated freeze/thawing, and the crude viral lysate was clarified by centrifugation at 3,500 rpm for 20 minutes. AAV particles were purified using double banding in a CsCl gradient and the presence of intact viral particles was confirmed using an electron microscope. AAV-2 titers in each preparation were approximately 10^9 (range 10^8–10^{10}) infectious particles. In preliminary tests, the purified AAV-2 particles were found to strongly bind anti-AAV-2 monoclonal antibody from clone A20 (American Research Products, Inc., Belmont, MA), and anti-AAV hyperimmune guinea pig serum (data not shown).

AAV-2 ELISA. A sandwich ELISA approach was chosen because it uses monoclonal antibody to bind specific antigen to reaction wells for better retention of AAV-2 particles in native form and reduced binding of nonspecific proteins (e.g., helper adenovirus proteins) [Carter et al., 1995]. Specifically, anti-AAV-2 monoclonal antibodies were coated overnight at a concentration of 1 μg/ml phosphate-buffered saline (PBS) and 4°C into each well of a 96-well microtiter plate (Corning, #25802). The plate was washed five times and incubated for 1 hr at 37°C with AAV particles diluted to 2.5 μg/ml in PBS, 0.5% milk, and 0.5% normal mouse serum. The wash step was repeated and serum samples diluted 1:100 in PBS and 0.5% milk were added to duplicate wells of the microtiter plate for 1 hr at 37°C. The plate was washed again prior to adding antihuman IgG conjugate diluted 1:4,000 in PBS for 1 hr at

TABLE I. Patient Characteristics and Results of DNA Hybridization Tests for β-Globin, HPV, and AAV in Cervical Tissue Samples

Subjects	Mean age (years)	Initial β-globin[a]	HPV DNA[b]	Second β-globin[c]	AAV DNA
Jamaican					
Normal (n = 94)	37	100%	14%	94%	None
CIN-1 (n = 105)	33	92%	44%	91%	None
CIN-3/CA (n = 92)	33	95%	93%	92%	None
U.S. university students (n = 79)[d]	23	99%	29%	98%	None

HPV, human papillomavirus; AAV, adeno-associated virus; CIN, cervical intraepithelial neoplasia.
[a]β-globin PCR results at the time of HPV DNA testing.
[b]Excluding individuals negative for amplification of β-globin.
[c]Results of repeat β-globin assays at the time of AAV DNA testing.
[d]All students had normal cytology, except one with CIN-1.

37°C, and the optical density was read in a microplate reader (Molecular Devices, Sunnyvale, CA) at a wavelength of 405 nm, with a reference wavelength of 490 nm. The geometric means of optical density levels in duplicate wells were used for analysis.

Statistical Methods

Categorical data were prepared in contingency tables and examined using Pearson's chi-square or the Mantel extension test, when evaluating trends. Continuous variables, such as ELISA optical density values, were first examined to assess their distributions, and appropriate data transformations were used to optimize their normality. These normalized continuous variables were studied using analysis of variance, when addressing overall group effects, and Tukey's HSD statistic, when addressing pairwise comparisons. We also estimated 95% confidence intervals around each group mean and median value. Associations between continuous covariates were assessed using multivariate linear regression. Standard commercial software were used in all analyses.

RESULTS

Patient characteristics and PCR results are shown in Table I. Essentially all patient specimens tested repeatedly positive for amplification of the β-globin gene, at the time of HPV DNA testing and again at the time of AAV DNA testing, indicating their adequacy for PCR. High prevalence of HPV DNA was also detected and, consistent with expectations, HPV prevalence increased monotonically with presence and grade of neoplasia.

AAV PCR dot blot findings are illustrated in Figure 1. Results for the AAV-2 plasmid titrations showed that both the A7/A8 and HAN-1/HAN-2 PCR assays detected 10 or more copies of the AAV genome. However, none of the 291 Jamaican clinic patients tested positive for amplification of AAV DNA in the A7/A8 PCR assay or in the HAN-1/HAN-2 PCR assay. Similarly, in 79 U.S. university students, we detected no amplification of AAV-DNA in any cervical specimens (Table I).

The failure to detect AAV DNA in cervical epithelial cells did not exclude the possibility of an inverse serologic association between AAV and cervical neoplasia, whether or not this finding might reflect a direct biologic relationship. To maximize the possibility of observing such an association, in addition to the Jamaican study subjects, we tested serum from U.S. patients with frank carcinoma of the cervix (n = 74) and noncancer control subjects (n = 77). Figure 2 shows optical density levels in the AAV-2 sandwich ELISA among the U.S. case-control subjects. Optical density levels were notably similar in cases and controls. Specifically, geometric mean (95% confidence interval) optical density was 0.141 (0.103–0.180) in cases and 0.125 (0.099–0.152) in controls. Median optical density values, first and third quartile levels, were also similar (Fig. 2), suggesting that no case-controls difference would be observed regardless of where a cut-off might be set. Although increasing age was significantly associated with higher AAV-2 antibody optical density levels (linear regression, P = .009), control for age did not affect the null relation of case-control status (ANOVA, P = .595) with AAV-2 humoral immune responses. Moreover, there was also no relation between AAV-2 serologic responses in the sandwich ELISA and presence or grade of neoplasia among the 291 Jamaican study subjects (ANOVA, P = .24).

DISCUSSION

Laboratory evidence suggests that AAV infection may interfere with HPV-induced tumorigenesis in vitro [Hermonat, 1994b; Walz et al., 1997]. However, the results of our investigation indicated that this relationship plays no role in the natural history of human cervical neoplasms. No AAV DNA was detected in any of the 370 cervical samples tested, though these specimens included all presumptive stages in the natural history of cervical tumorigenesis—from normal, HPV-negative epithelium through HPV-positive invasive cervical cancer. Moreover, the absence of AAV DNA in any specimens suggests that AAV does not infect cervical epithelial cells in vivo, or does so at low prevalence. Serology, similarly, showed no relation between cervical neoplasia and AAV infection.

The negative DNA hybridization findings cannot be ascribed readily to laboratory methods. The sensitivity of HAN-1/HAN-2 and A7/A8 PCR were each demonstrated to be approximately 10 copies of the AAV genome and, because the primer sets amplify different

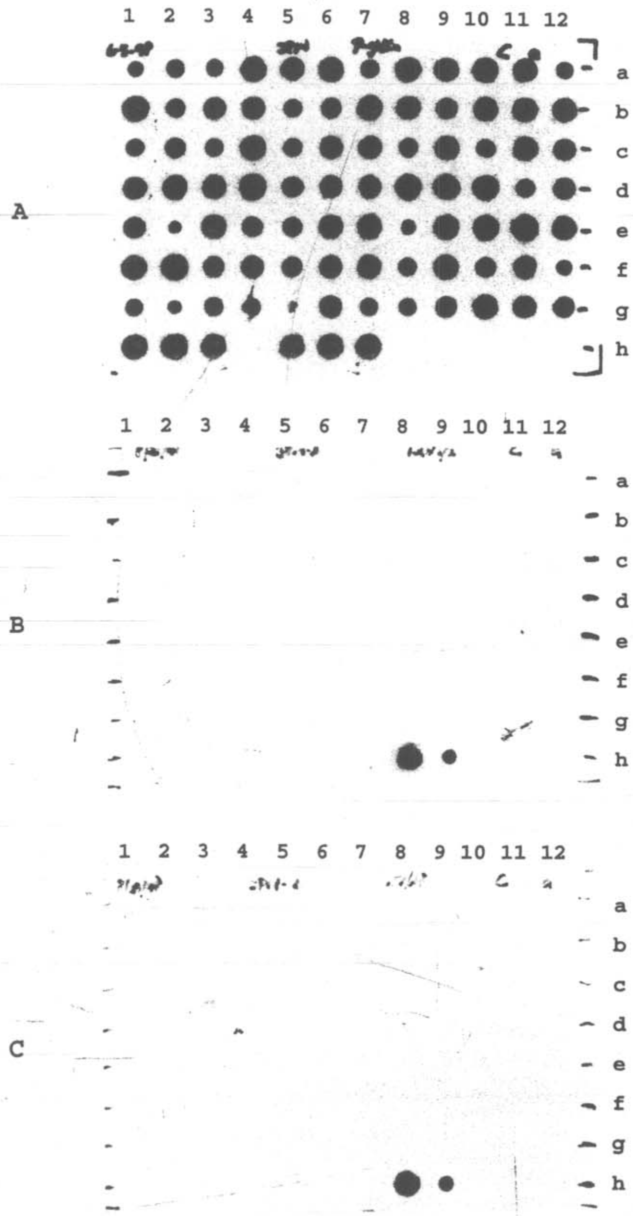

Fig. 1. Adeno-associated virus (AAV) polymerase chain reaction (PCR) dot blot results in representative specimens. Three nylon filters are shown, each containing amplification products from the same 83 specimens hybridized with biotinylated probes. Filter **(A)** has products from β-globin primers PC04/GH20 hybridized with PC03 probe. Filter **(B)** has products from AAV-2 primers HAN-1/HAN-2. Filter **(C)** has products from AAV primers A7/A8. Each AAV-2 product was hybridized with the appropriate oligonucleotide probe described in the text. The eight wells in column 7 are β-globin positive, AAV negative human cells (K562). Row H8 through H12 are 10-fold titrations of AAV-2 plasmid form 100 to <1 copy of an AAV-2 plasmid. Eighty-two of the 83 clinical specimens were β-globin positive (A). Both AAV-2 probes detected 10 copies of AAV sequences (B and C). However, AAV DNA was not detected in any of the clinical specimens (B and C).

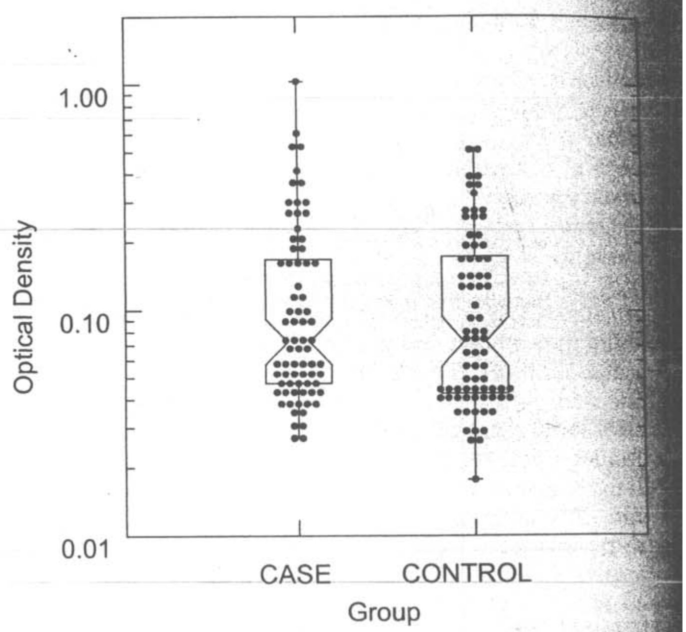

Fig. 2. Immunoglobulin G (IgG) responses to adeno-associated virus type 2 (AAV-2) particles in cervical cancer cases and controls. The values shown are optical density results in a sandwich enzyme-linked immunosorbent assay (ELISA) test. Boxplots are interpreted as follows: the waist is the median, diagonal lines indicate 95% confidence intervals about the median, lower and upper horizontal lines indicate 25th and 75th percentiles of the distribution, and upper and lower bars indicate the range of the data excluding outliers.

viral genes, the results are unlikely to reflect loss of selected viral sequences (e.g., during integration into host DNA). Furthermore, the adequacy of the tissue specimens for PCR was demonstrated by the repeated amplification of human β-globin DNA, and by the detection of HPV DNA, which showed the expected strong positive relation with presence and grade of neoplasia, using these same materials. The satisfactory HPV DNA and β-globin results also make it unlikely that DNA extraction procedures or inhibitors of PCR could explain the failure to detect AAV DNA sequences. Lastly, patient characteristics are unlikely to explain the absence of detectable AAV DNA in the cervix, as the findings were confirmed in specimens obtained from two substantially different groups, clinic patients in Kingston, Jamaica and U.S. university students.

To further assess exposure to AAV in study subjects, an AAV-2 sandwich ELISA was developed using whole virus particles. During assay development, successful growth of AAV was demonstrated by immunofluorescence and confirmed by electron microscopy. Initial testing using monoclonal antibodies and hyperimmune animal serum demonstrated that these particles could bind AAV-2 antibodies. The sandwich ELISA approach controls nonspecific reactivity by using monoclonal antibody to preferentially bind the relevant antigen to reaction wells [Carter et al, 1995]. However, we found no relation between AAV-2 antibody responses and cervical neoplasia among U.S. cervical cancer case-control subjects or in Jamaican study subjects.

Therefore, neither DNA hybridization methods nor serology suggested any relation of AAV with cervical neoplasia. We cannot easily explain the differences between our observations and those in some earlier studies [Tobiasch et al., 1994; Han et al., 1996; Walz et al., 1997]. It must be stressed, however, that our findings are based on the cumulative results of multiple complementary laboratory approaches, assessed individually for their validity, and testing in several different patient groups, using specimens collected by independent research teams. Resolution of this matter may require interlaboratory studies using masked, third-party-prepared replicate specimens, which include laboratory-prepared positive and negative controls to directly measure PCR sensitivity and specificity. Nonetheless, the findings in the current study are clear. Our data suggest that AAV plays no role in the natural history of cervical tumorigenesis, and that AAV does not commonly infect cervical epithelial cells.

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